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ORIGINAL ARTICLE

Skin-Protective and Anti-inflammatory Effects of *Hibiscus syriacus* L. (Mugunghwa): A Comparative Study of Five Parts of the Plant

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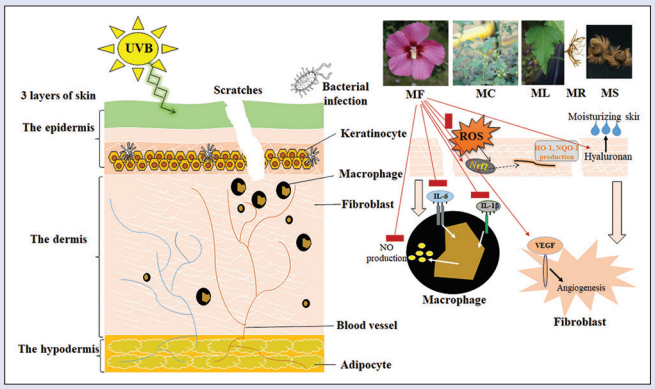
ABSTRACT

Aim: The skin barrier is vulnerable to internal and external elements. However, certain plant extracts have promise for protecting the skin. *Hibiscus syriacus* L. is a Korean national plant (mugunghwa) that has been traditionally used to treat some skin diseases; however, its pharmacological activities are poorly understood. The present study has evaluated the skin-protective effect of mugunghwa flowers (MF), cortexes (MC), roots (MR), leaves (ML), and seeds (MS) in external factor-stimulated human epidermal keratinocytes (HaCaTs), normal human dermal fibroblasts (NHDFs), and a widely used murine macrophage cell line (RAW 264.7 cells). **Materials and Methods and Results:** The active components were identified as palmitic acid and linoleic acid by high-performance liquid chromatography analysis. The parts of mugunghwa had low 2,2-diphenyl-1-picrylhydrazyl scavenging activities but moderate 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt scavenging capacity. All parts' extracts at 100 μg/mL decreased nitric oxide production in inflammation-induced RAW 264.7 cells and mRNA expression of the cytokines interleukin-6/1β and the enzymes inducible nitric oxide synthase/cyclooxygenase-2. On HaCaTs, MF, MC, and MR (10–50 μg/mL) significantly increased hyaluronan production over that of controls. Moreover, MF activated NAD (P) H:quinone oxidoreductase 1 and heme oxygenase-1 and inhibited intracellular reactive oxygen species production. Treatment with MF, MC, MR, ML, and MS (10 μg/mL) reduced the scratching wound site in NHDFs to be considered by vascular endothelial growth factor signaling activation. **Conclusion:** Taken together, among all the five parts of the mugunghwa plant, MF effectively inhibited lipopolysaccharide, ultraviolet B irradiation, and scratching wound-induced inflammation in skin cells.

Key words: *Hibiscus syriacus* L, hyaluronan, skin inflammation, ultraviolet B, wound

SUMMARY

- In summary, the data demonstrated that mugunghwa flowers (MF) most effectively protected skin cells against inflammatory damage, followed by mugunghwa cortexes, among the extracts of the five parts of *Hibiscus syriacus*. Although MF had low 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt scavenging activities, MF at 10 and 50 μg/mL increased hyaluronan secretion and decreased reactive oxygen species production in human epidermal keratinocytes and it decreased nitric oxide production as well as the expression of interleukin-6/1β and inducible nitric oxide synthase/cyclooxygenase-2. The better anti-inflammatory effects of MF can be partly explained by the higher contents of two active ingredients, palmitic acid and linoleic acid. It is necessary to assess the effect of MF and the underlying mechanisms *in vivo* in further studies.



Abbreviations used: *Hibiscus syriacus* L.: *H. syriacus* (mugunghwa); MF: Mugunghwa flowers; MC: Mugunghwa cortexes; ML: Mugunghwa leaves; MR: Mugunghwa roots; MS: Mugunghwa seeds; HaCaTs: Normal human keratinocytes; NHDFs: Normal human dermal fibroblasts; RAW 264.7 cells: Murine macrophage cell line; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; NO production: Nitric oxide production; LPS: Lipopolysaccharide; IL-6/IL-1β: Interleukin-6/1β; iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase-2; NQO-1: NAD (P) H:quinone oxidoreductase 1; HO-1: Heme oxygenase-1; ARE: Antioxidant response element; ROS: Reactive oxygen species; VEGF: Vascular endothelial growth factor.

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INTRODUCTION

Hibiscus syriacus L. is the most popular plant in Korea, with a national history dating back to the Silla Kingdom (57 B.C.) Its international common name is the rose of Sharon, with various subspecies [Figure 1a]. *H. syriacus* is known to Koreans as "mugunghwa," meaning

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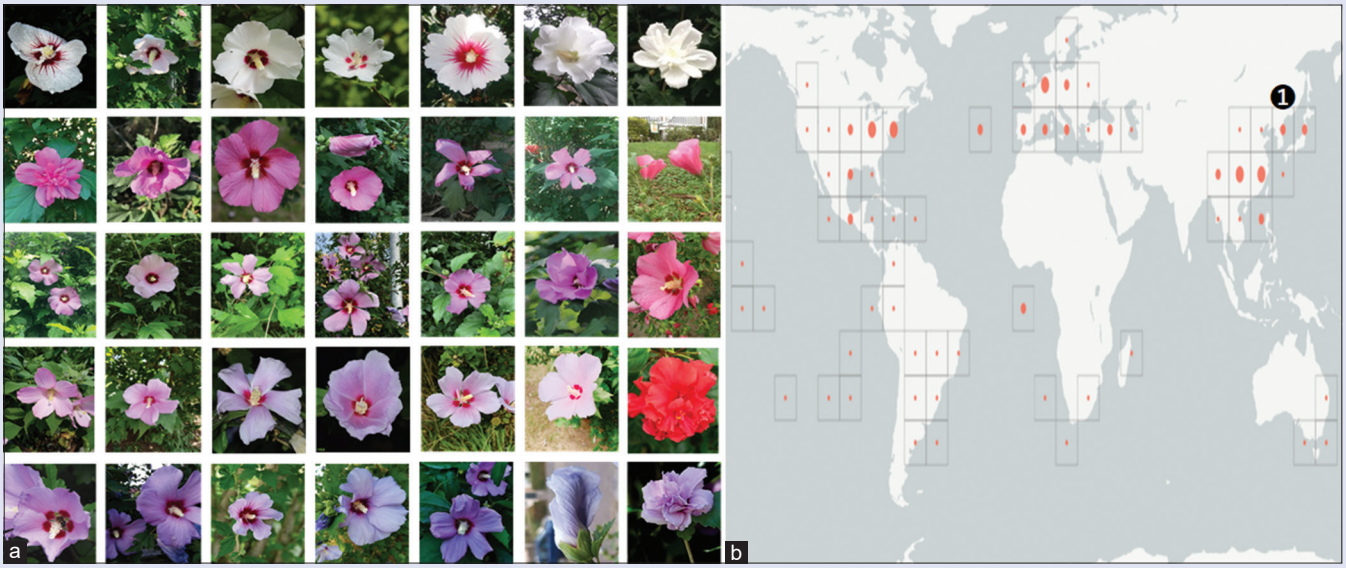

Figure 1: Worldwide distribution of *Hibiscus syriacus* L. (a) and the diversity of its flowers (b). Note: 1, Republic of Korea. Refs: https://plants.usda.gov/core/profile?symbol=HISY; https://www.gbif.org/occurrence/gallery?taxon_key=3152583; https://ko.wikipedia.org/wiki/%ED%8C%8C%EC%9D%BC: Hibiscus_syriacus_red.jpg; https://www. 123rf. com/photo_84610984_red-hibiscus-hibiscus-syriacus-it-s-famous-flower-to-paint-in-textile.html

"eternity."[1] According to an ancient book of Korean medicinal herbs (Dongeuibogam 1613), it can be used to treat fever, insomnia, and some internal diseases.[2] Although the Korean government has been devoting resources to grow and maintain mugunghwa plants throughout the country with well-developed cultivation methods, only a few studies have investigated their pharmacological activities. Even though Korean people have known about the medicinal properties of the barks and flowers of this plant since the 16th century, most publications have focused on flower pigmentation or plant cultivation.[3]

Hibiscus species were widely distributed from tropical lands including Asia, the Middle East, Europe, and North America [Figure 1b].[4] Among over 300 species of the genus *Hibiscus*, *Hibiscus sabdariffa* and *Hibiscus rosa-sinensis* were the main species investigated for pharmacological activities.[5] Various parts have been used in oriental medicine, including flowers, hibisci cortexes (root and stem barks), roots, leaves, and seeds. Bioactive compounds including flavonoids, anthocyanins, phenolic acids, lignans, coumarins, hydroxyhibiscone A, nonanoic acid, triterpene derivatives, and polysaccharides have been isolated from *Hibiscus* species.[6] The antioxidant activities of the anthocyanins from *Hibiscus* flower pigments have been examined over the past 20 years [Table 1].

Skin inflammation can be easily caused by internal factors (hormones, natural aging, mutated genes, or other miscellaneous disease-related signaling pathways) and external factors (ultraviolet [UV] radiation, microbial infection, chemicals, and free radicals). Inflammation is a rapid response of the skin tissue against those factors, in which inflammatory mediators such as cytokines are secreted from skin cells including keratinocytes, fibroblasts, and macrophages.[7] Many *in vitro* studies were designed to study proinflammatory factors relevant to cellular conditions related to the disease of interest's symptoms and causes.[8] The reaction patterns of the cells from the different skin layers (the epidermis, the dermis, and the hypodermis) vary according to the severity and nature of any given stimulus. For inflammation stage, neutrophils and macrophages migrating to the wound site can be modeled by the most typical inflammatory experiment with lipopolysaccharide (LPS) (bacterial LPS), a main constituent of the membrane of Gram-negative bacteria.[9] In murine macrophage cell line (RAW264.7 cells), LPS mimics infectious microbes causing inflammation through inducing high nitric oxide (NO) production and stimulating the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and proinflammatory cytokines (interleukin-1β [IL-1β] and IL-6) in host responses.[10]

Table 1: Pharmacological activities of *Hibiscus* species

Hibiscus species	Pharmaceutical activities with references (PMID number)
Hibiscus syriacus	Anticancer (9542172; 18306460; 24862067; 25885960), antioxidant (10617409; 11575966; 12647536), antibacterial (Punasiya *et al*., 2011; Punasiya and Pillai, 2014), anti-aging (20798580; 30571966), antifungal activities (739389; 22870060)
Hibiscus sabdariffa	Antioxidant (10762726; 25525823; 11902968; 19330881), anticancer (16018963; 18030661; 25945622), anti-tyrosinase and antibacterial (Wong *et al*., 2010), amylase inhibitory (10879476, 23216107), adipogenesis inhibitory (14499025), anti-inflammatory (12491040, 26213025, 30381729), antiparasitic (27118499), antiviral (26143492), inhibitor of starch digestion (11676026), immunosuppressive activities (Lubega *et al*., 2013)
Hibiscus cannabinus	Antioxidant activity (23099617), anti-cancer (25183141), anti-inflammatory activities (Chaudhari *et al*., 2015)
Hibiscus mutabilis	Antioxidant, antibacterial, anti-tyrosinase (Wong *et al*., 2010), anti-inflammatory and wound healing activities (24653565)
Hibiscus rosa-sinensis	Antioxidant and antibacterial (23569938, 24811803), analgesic and anti-inflammatory (30150191), anti- skin cancer activities (28053887)

PMID: PubMed IDentifier

Inflammatory stimuli are reactive oxygen species (ROS) inducers which activate the NF-E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway, a key modulator maintaining intracellular redox balance.[11,12] ROS inducers include injuries or exposure of radiation (UVA, UVB, γ-rays, or X-rays), heavy metals, and other chemical compounds; all of these can change cellular oxidation balance and inflammatory repair status.[13] In the host response, the anti-inflammatory signaling pathway causes fibroblasts to proliferate and migrate to the wound area and causes the secretion of new extracellular matrix to form granulation tissue. Vascular endothelial growth factor (VEGF) is a key contributor in the formation of angiogenesis which is an important part of the proliferative phase of the wound healing process.[14]

In this study, the effects of five parts of the *H. syriacus* plant were investigated with different stimuli (LPS, dimethylsulfoxide (DMSO), UVB radiation, and scratching) on cells involved in skin formation and the inflammatory response.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM) media and fetal bovine serum (FBS) were purchased from HyClone, Seoulin Bioscience (Seoul, Korea). Trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05%) with phenol red and antibiotics were purchased from Gibco BRL (Grand Island, USA). A Hyaluronan and VEGF DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH, 97%), methylthiazolyldiphenyl-tetrazolium bromide (MTT), L-ascorbic acid (99%), allantoin (≥98%), lipopolysaccharides from *Escherichia coli* O55:B5, Griess reagents A and B, sodium nitrite ($NaNO_2$, ≥97%), dexamethasone (DEX, ≥97%), and 2',7'-dichlorofluorescein diacetate (DCFH-DA, ≥97%) were purchased from Sigma-Aldrich (Korea). The primary antibodies against β-actin, NAD (P) H:quinone oxidoreductase 1 (NQO-1), and heme oxygenase-1 (HO-1), and the secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and Cell Sciences (Canton, USA). SPLScar scratchers (HDPE scratcher, 24-well lid) were purchased from SPL Life Sciences (Seoul, Korea). Human epidermal keratinocytes (HaCaTs) and normal human dermal fibroblasts (NHDFs) were purchased from ScienCell Research Laboratories (California, USA), and the murine macrophage-like cell line RAW 264.7 was purchased from the ATCC (Virginia, USA).

Preparation of mugunghwa extracts

Dried *H. syriacus* L. flowers, cortexes, leaves, roots, and seeds were powdered and extracted with water, hexane, or 50%–100% ethanol for 24 h at 25°C–27°C with three times repetition. The samples were then transferred with a filtration paper into a new flask and evaporated under vacuum until it reaches the drying level. The 100% hexane extract of flowers and seeds, the 70% ethanol extracts of cortexes and leaves, and the 80% ethanol extract of roots were chosen for further study with 10–100 μg/mL by NO production assay.

High-performance liquid chromatography analysis

A Dionex Chromeleon™ chromatography data system was used with P580 and UVD100 detectors (Thermo Fisher Scientific Inc., Waltham, MA, USA). A Watchers 120 ODS-AP C_{18} column was 250 mm × 4.6 mm and 5 μm particle size (Daiso Corp., Japan). The solvents were 0.2% formic acid (A, v/v) and acetonitrile (B) with the following elution profile: 0–5 min, 10% B; 5–30 min, 10%–22% B; 30–32 min, 22%–90% B; 32–37 min, 90% B; 37–39 min, 90%–10% B; and 39–46 min, 10% B. The flow was run with 1 mL/min at a 320 nm wavelength and 25°C.

Cell culture

HaCaT, NHDF, and RAW 264.7 cells were seeded in 24-well plate at cell densities of 1×10^4, 2×10^4, and 1×10^5 cells/cm^2, respectively. All cell lines were cultured in DMEM supplemented with 10% FBS and 1% antibiotics at 37°C in a humidified atmosphere with 5% CO_2. The subculture of HaCaTs and NHDFs was carried out with trypsin-EDTA while RAW 264.7 cells were subcultured using a scraper (SPL Life Science Co., Korea).

Antioxidant capacity

The antioxidant capacity of mugunghwa extracts was measured by DPPH and ABTS tests.[15] In brief, AAPH solution (1 mM) and ABTS (2.5 mM) were mixed in phosphate-buffered saline (PBS) (100 mM, pH 7.4) containing 150 mM NaCl. The mixture was incubated at 70°C for 30 min and cooled at room temperature for 10 min. The radical $ABTS^+$ in PBS (pH 7.2) has an absorption of 0.650 ± 0.02 units at 734 nm.

For DPPH assay, 20 μL of extract (final concentrations: 1, 10, 50, 100, and 250 μg/mL) and 180 μL DPPH (final concentration: 0.2 mM) were added per well to a 96-well plate and incubated for 30 min at 37°C in the dark. The absorbance was read at 517 nm.

In both assays, the same concentration range of ascorbic acid (1, 10, 100, 250, and 500 μg/mL) was used as an antioxidant standard.

Cell viability

The cell viability was assayed as described previously.[16] After 24 h of sample treatment, MTT (5 mg/mL) was put in cell culture plates (final concentration 100 μg/mL) for 2 h. Before measurement, the supernatants were discarded and DMSO was added to form the violet crystals. It was measured at 570 nm using a microplate reader (Molecular Devices, USA).

Nitric oxide assay in lipopolysaccharide-induced RAW 264.7 cells

The NO production inhibitory effects of mugunghwa extracts were examined in RAW 264.7 cells. Cells were seeded in 96-well culture plates and incubated for 24 h. The cells were treated with the extracts (10, 50, and 100 μg/mL) and LPS (1 μg/mL) at the same time dissolved in serum-free media for 24 h. The NO production of cell culture supernatants was measured with Griess reagents. A range of concentrations of $NaNO_2$ with 100 μM as the highest concentration was used to create the standard curve at 540 nm.[17]

Reverse transcription-polymerase chain reaction

The reverse transcription-polymerase chain reaction (RT-PCR) technique was applied to measure the expression levels of IL-6, IL-1 β, iNOS, and COX-2. Briefly, total RNA was isolated from cells with TRIzol reagent (Invitrogen Life Technologies, CA, USA). cDNA template was used together with PCR PreMix (Bioneer). Primer sequences were referred from previous studies including IL-6,[18] IL-1β,[19] iNOS,[20] GAPDH,[21] and COX-2 (designed by Primer3 with forward 5'-TGC TGG AAA AAC CTC GT-3'and reverse 5'-AAA ACC CAC TTC GCC TCC AA-3'). RT-PCR was performed as described in Ngo *et al.*[15] Products were stained with nucleic acid staining solution (NobleView Co., Korea) and then run by agarose gel electrophoresis. Data were read under UV illumination.

Measurement of hyaluronan secretion

Ten percent DMSO served as a negative control. The secretion of hyaluronan in cell culture supernatants was determined using a Hyaluronan DuoSet ELISA kit following the manufacturer's instructions. In brief, the plate preparation was sealed with capture reagent overnight and blocked by 300 μL of reagent diluent for 1 h at room temperature. Samples or standards were diluted in reagent diluent and incubated for 2 h. Hyaluronan concentration was measured using Streptavidin-HRP-combined detection reagent and substrate/stop solution. The absorbance wavelength was set from 450 to 540 nm.[22]

Ultraviolet B-induced reactive oxygen species production and antioxidant elements in keratinocytes

Cells were subjected to UVB irradiation at 70%–80% confluence. UVB source was provided at 100 mJ/cm^2 for 25 s using a Bio-Link BLX-312 machine (Vilber Lourmat GmbH, France). After that, cells were incubated for 24 h with 10, 50, or 100 μg/mL of mugunghwa extract in serum-free medium. The intracellular ROS production was measured by staining cells with 30 μM DCFH-DA for 30 min at 37°C. Data were analyzed by a flow cytometry BD Accuri C6 (CA, USA).

Western blot analysis

HaCaTs were harvested after UVB irradiation for 3 h (for detection of NQO-1 and HO-1) and extracted proteins using a lysis buffer. Protein concentration was measured using the Bradford reagent (Bio-Rad, Hercules, CA, USA), separated by sodium dodecyl sulfate–polyacrylamide gel, and transferred using a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Proteins were determined using chemiluminescence detection ECL reagents (Fujifilm, Japan) and ImageMaster™ 17 2D Elite software, version 3.1 (Amersham Pharmacia Biotech, USA).

In vitro wound scratch assay

The wound healing migration rates of human dermal fibroblasts were assessed by the scratch assay method. The cells were seeded at 2×10^4 cells/cm^2 into a 24-well plate. After 24 h of incubation, the 90%–100% confluent cells were scraped horizontally with a sterile SPLScar scratcher (SPL Life Sciences, Gyeonggi, Korea). The debris was removed by washing with PBS. The cells were treated with mugunghwa extracts at a range of concentrations (10, 50, and 100 μg/mL) in serum-free media. Allantoin (10 μg/mL) was used as a positive control (PC). The scratch, which represented a wound, was photographed at 0, 6, 12, 18, and 24 h using phase-contrast microscopy at ×10 magnification. The images were analyzed using Axio Observer D1 Inverted Phase Contrast Microscope (Carl Zeiss, Germany) to determine the wound healing rate (% of control). Experiments were performed in a triplicate manner.

After 24 h of scratching, the secretion of VEGF in cell culture supernatants was determined using a VEGF DuoSet ELISA kit following the manufacturer's instructions. The absorbance wavelength was set from 450 to 540 nm.[23]

Statistical analysis

All values were calculated as mean ± standard error with triplicated experiments. Data were subjected to analysis of variance using the Tukey's test to analyze intergroup differences. Statistical significance was set at $P < 0.05$.

RESULTS

Analysis of mugunghwa extracts

The mugunghwa flowers (MF), cortexes (MC), roots (MR), leaves (ML), and seeds (MS) were extracted from the dried forms. As shown in Figure 2, palmitic acid and linoleic acid were the major components in the mugunghwa extracts. These fatty acids were detected in the MF, MC, ML, MR, and MS extracts, but MF had the highest palmitic acid and linoleic acid content: 14.1% and 10.3%, respectively.

Antioxidant activity of mugunghwa extract

Ascorbic acid as a PC had the highest DPPH and ABTS scavenging activities, with IC_{50} values of 7.8 ± 0.9 μg/mL and 4.2 ± 1.4 μg/mL, respectively. ML and MR had the highest DPPH and ABTS antioxidant activities among the five types of extracts. As shown in Figure 3, ML had the highest DPPH scavenging activity with an IC_{50} of 130.2 ± 23.9 μg/mL, followed by MR (189.2 ± 24.5 μg/mL) and MC (199.2 ± 32.9 μg/mL). ML and MR had approximate IC_{50} values of 400 μg/mL for inhibition of ABTS radicals.

Lipopolysaccharide -induced inflammatory response in RAW 264.7 cells

The mugunghwa extracts did not significantly affect the survival of RAW 264.7 cells [Figure 4a and b]. They not only improved macrophage viability but also aided in inhibiting NO production. Treatment of DEX (PC) had a NO production decrease by up to 50% while the NO production decreased by 96.4% with MF, 82.0% with MC, 50.7% with ML, 83.8% with MR, and 69.0% with MS (at 100 μg/mL).

Based on its inhibition of NO production, MF was chosen as the best extract among the five mugunghwa extracts for further study. The expression levels of the inflammation-related cytokines IL-6/IL-1β and iNOS/COX-2 were measured by RT-PCR. These levels were significantly elevated after LPS treatment but decreased after treatment with MF (100 μg/mL) [Figure 4c and d].

Mugunghwa and hyaluronan expression in epidermal keratinocytes

The effect of mugunghwa extracts on cell viability was investigated in HaCaT cells with an MTT assay. As shown in Figure 5a and b, the viabilities of ML (at 100 μg/mL)-treated cells were significantly decreased compared to nontreated cells by up to 39.5%, and all of the plant part extracts were used with two concentrations at 10 and 50 μg/mL. Treatment with MF, MC, MR, and MS (at 10 μg/mL) slightly enhanced the cell viability.

In measurement of the expression level of hyaluronan, retinoic acid significantly increased the secretion of hyaluronan to a level that was 358.7% greater than that of the nontreated group. The extracts from all parts of the mugunghwa plant were associated with enhancement of hyaluronan production: 10 μg/mL of MF, MC, ML, MR, and MS promoted hyaluronan expression by 157.6%, 139.1%, 150.8%, 123.6%, and 120.7%, respectively.

Ultraviolet B-induced reactive oxygen species production and antioxidant elements in keratinocytes

In Figure 5c-f, ROS levels were significantly increased ($P < 0.01$) in UVB-irradiated cells compared with nonirradiated cells resulting to expression of NQO-1 and HO-1.As expected, the antioxidant element molecules including NQO-1 and HO-1 were activated by UVB irradiation via ROS regulation. The analysis of NQO-1 and HO-1 band intensities

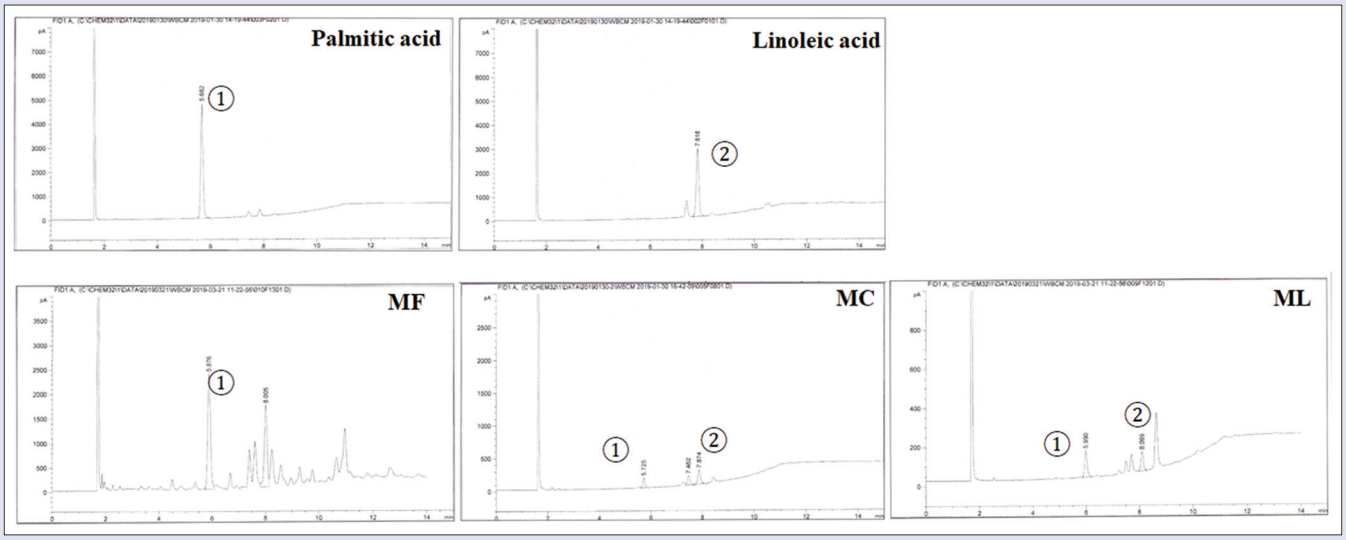

Figure 2: High-performance liquid chromatography results for palmitic acid standard, linoleic acid standard, and mugunghwa extracts. Peak 1, palmitic acid; Peak 2, linoleic acid. MF: Mugunghwa flowers; MC: Mugunghwa cortexes; ML: Mugunghwa leaves

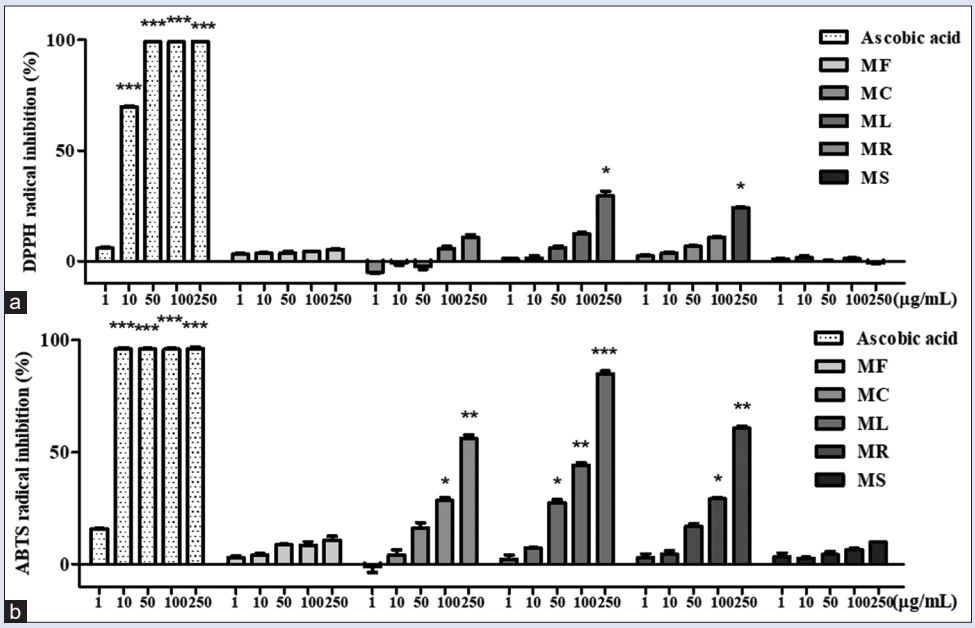

Figure 3: The antioxidant capacity of mugunghwa extracts. (a) 2,2-diphenyl-1-picrylhydrazyl and (b) 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging assays. The concentrations of mugunghwa extracts and ascorbic acid were 1, 10, 50, 100 and 250 μg/ml. Ascorbic acid was used as a positive control. Values are mean ± standard deviation. MF: Mugunghwa flowers; MC: Mugunghwa cortexes; ML: Mugunghwa leaves; MR: Mugunghwa roots; MS: Mugunghwa seeds

was shown that the MF-treated group had a 65.2% ROS reduction and 135.9%–212.2% ARE elevation compared to the UVB group.

In vitro wound scratch assay

The effect of mugunghwa extracts on cell viability was investigated in NHDF cells by MTT assay. As shown in Figure 6, the viabilities of ML (at 50–100 μg/mL)-treated cells were significantly decreased ($P < 0.001$) compared to nontreated cells, by up to 49.5%, which was similar to the viability data of HaCaT cells. Treatment with MF, MC, MR, ML, and MS at 10 μg/mL did not decrease cell viability compared to nontreated cells.

The wound site was decreased by 21.5% in nontreated cells and by 1.7% in the allantoin (10 μg/mL)-treated cells. Notably, treatments with MF, MC, ML, MR, and MS led to healing rates (diminishing the wound area) better than that seen in the PC group. MF, MC, and MR possessed a 38.9%, 36.0%, and 49.5% reduction of wound site, respectively. It is supposed due to their abilities of VEGF signaling activation, as shown in Figure 6c.

DISCUSSION

In this study, the anti-inflammatory effect of five *H. syriacus* L. extracts including flowers, cortexes, leaves, roots, and seeds was investigated.

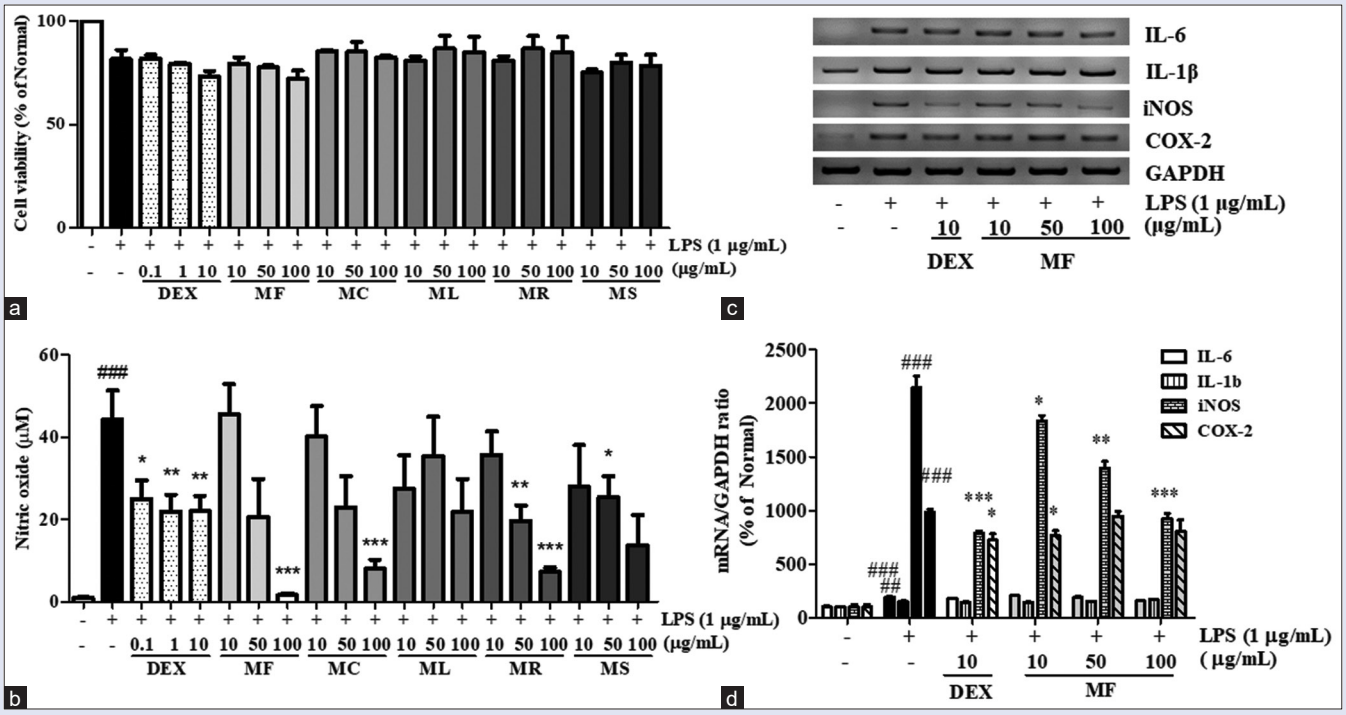

Figure 4: Effects of mugunghwa extracts on nitric oxide production and inflammatory cytokine expression in lipopolysaccharide-induced murine macrophage cell line. (a) RAW 264.7 cell viability; (b) nitric oxide production; (c) the mRNA levels of inducible nitric oxide synthase, interleukin-6/1β, and cyclooxygenase-2 with GAPDH as an internal control; (d) analysis of mRNA EXPRESSION Cell inflammation was induced by treatment with lipopolysaccharide at 1 μg/mL. Dexamethasone at 0.1, 1, and 10 μg/mL was used as a positive control. All data are shown as the mean ± standard deviation of at least three independent experiments. Number signs indicate highly significant differences from control cells. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Asterisks indicate highly significant differences from controls. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

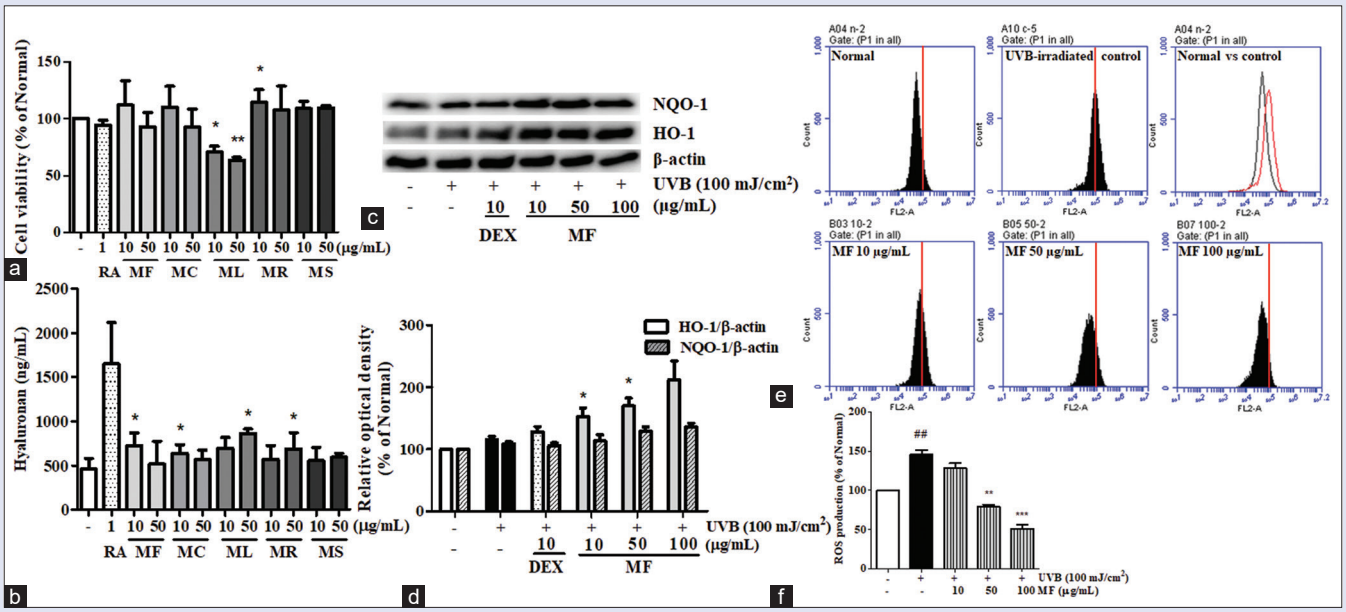

Figure 5: Effects of mugunghwa extracts on hyaluronan production and cellular antioxidants/oxidants of keratinocytes. (a) Cell viability was measured by the methylthiazolyldiphenyl-tetrazolium bromide assay. Absorbance was measured at 570 nm using a microplate reader; (b) hyaluronan production (ng/mL) was assessed by measuring the absorbance at 450–540 nm using a microplate reader; HaCaTs were treated with different concentrations of mugunghwa flower, mugunghwa cortex, mugunghwa leaf, mugunghwa root, and mugunghwa seed extracts (10 and 50 μg/mL) for 24 h. Dexamethasone at 10 μg/mL was used as a positive control. (c) The protein levels of antioxidant elements including NAD (p) H:quinone oxidoreductase 1 and heme oxygenase-1 with β-actin as an internal control; (d) analysis of protein expression; (e) intracellular reactive oxygen species production in flow cytometry signals; and (f) analysis of reactive oxygen species production. HaCaTs were irradiated or sham-irradiated with ultraviolet B (100 mJ/cm²), followed by treatment with different concentrations of mugunghwa flower extract (10, 50, and 100 μg/mL) for 24 h. All data are shown as the mean ± standard deviation of at least three independent experiments performed in triplicate

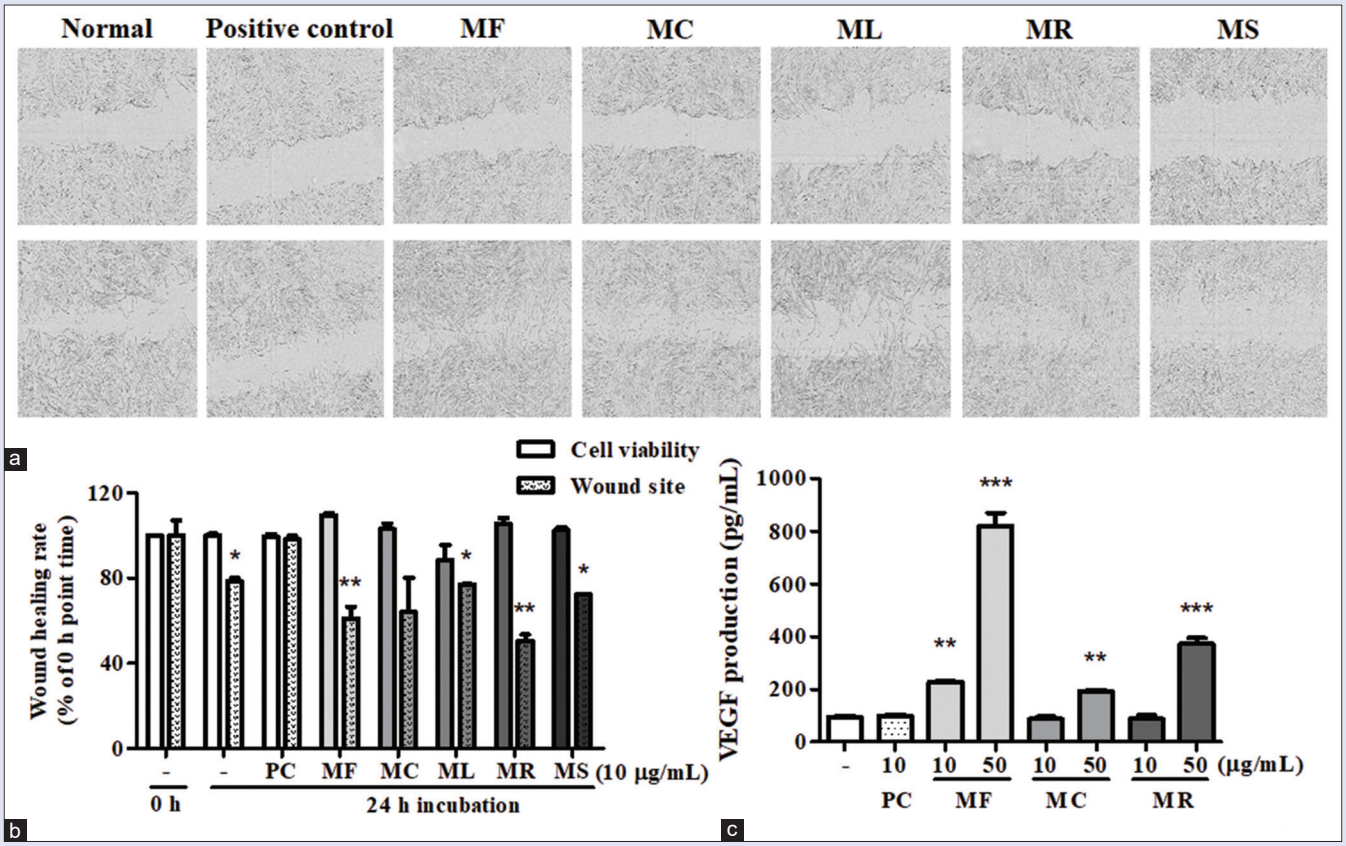

Figure 6: Number signs indicate highly significant differences from control cells. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Effects of mugunghwa extracts on wound healing. (a) Pictures of wound healing at 0 h and 24 h time points. (b) Normal human dermal fibroblast cell viability and wound healing rate. (c) Vascular endothelial growth factor production. Allantoin at 10 μg/mL was used as a positive control. All data are shown as the mean ± standard deviation of at least three independent experiments

In vitro inflammation experiments with three cell lines showed that the flower extracts had the highest anti-inflammatory effects and the richest palmitic and linoleic acid contents (14.1% and 10.3%). The flower extracts of other *Hibiscus* species have also shown anti-inflammatory effects.[24,25] Kim *et al.* reported that the $CHCl_3$ and water extracts of *H. syriacus* roots inhibited the UV irradiation-induced degradation of collagen.[26] Treatment with its flowers increased HaCaT cell migration and proliferation.[27] In their studies, palmitic acid (18.9%) and linoleic acid (8.6%) were detected in the flower preparations. In our data, the linoleic acid content was slightly higher (10.3%). On the other hand, other phenolic compositions of *Hibiscus* species vary along their parts. For instance, *Hibiscus* flowers can be rich in lutein, chlorogenic acid, quercetin, ferulic acid, rutin, anthocyanins, and flavonoids (gossypetin, hibiscetin, and sabdaretin).[28-32] The leaves of *Hibiscus* species can contain chlorogenic acid, quercetin, rutin, kaempferol, and catechin hydrate or ellagic acid.[33-36] However, the stems, roots, stem and root barks, or hibisci cortexes were detected with some other phenolic compounds such as daidzein, genistein, hibiscuside, syringaresinol, coumarino-lignan, scopoletin, and cleomiscosins A, C, and D.[5] These different phytochemical profiles of the parts of *H. syriacus* can explain their different anti-inflammatory effects.

The present data also demonstrated that the cortexes, leaves, roots, and seeds of mugunghwa possessed moderate anti-inflammatory activities. Similarly, Shin and Ha confirmed that *H. syriacus* possessed antioxidant activity and significantly improved blood circulation.[37] Recently, the root of *H. syriacus* was observed to have antidepressant, neuroprotective, anti-inflammatory, and antiaging effects.[38,39] It might inhibited the expression of nuclear transcription factor-kappa B and MAPK/ATF2 in LPS-stimulated RAW264.7 cells and enhanced the expression of type I procollagen and filaggrin in UVB-irradiated mouse skin. Di Martino *et al.* described the healing and hydration properties of an *H. syriacus* ethanolic extract.[40] We also investigated the antiaging and anti-wrinkle effects of hibiscus cortex extract in a previous study.[1] These effects were thought to be due to the palmitic and linoleic acid contents. In addition to those compounds, many biologically active compounds from the genus *Hibiscus* were reported in a review by Vasudeva and Sharma.[5]

Although the DPPH and ABTS scavenging activities of mugunghwa extracts were quite low (IC_{50} 100–200 μg/mL), their anti-inflammatory effects as well as their inhibitory effects on NO production were still high. Similar to the mugunghwa extracts, *Aralia elata*, some fruits and vegetables, different varieties of *Sanghuang* species and 44 Chinese medicinal plants have the same DPPH and ABTS scavenging activity levels (IC_{50} 100–200 μg/mL) and also have strong anti-inflammatory effects.[41-44] The restoring of the redox balance has been shown after treatment with mugunghwa extracts in terms of Nrf2/ARE signaling in response to ROS. This result means that mugunghwa could care for damaged skin which is exposed to the sun. Mugunghwa extracts can, therefore, display a role as antiaging wonders for the skin by fighting free radicals helping to rejuvenate damaged skin cells.

All five parts of the mugunghwa plant can enhance the secretion of hyaluronan in keratinocytes. Hyaluronan is a ubiquitous polysaccharide in both the epidermis and the dermis that belongs to the

glycosaminoglycan family; it is essential for maintaining viscosity and skin moisture.[45] External stimuli such as UV radiation and chemical exposure can reduce the production of hyaluronan, resulting in dry skin; however, mugunghwa extracts can be an alternative to hyaluronan for moisturizing the skin. Therefore, mugunghwa extracts enhance water retention in skin and relieve dehydrated skin.

Inflammation has three stages: (1) tissue inflammation and signal transmission occur; (2) tissue forms through myofibroblast accumulation, extracellular matrix synthesis, angiogenesis, re-epithelialization, and tissue remodeling; and (3) tissue maturation and remodeling promotes wound closure and completes tissue regeneration. In this study, the anti-inflammatory effects of mugunghwa extracts were assessed in terms of their inhibition of NO production. LPS was used to activate RAW 264.7 macrophage cells, which then produced NO and expressed IL-6, IL-1β, iNOS, and COX-2. The oversecretion of these cytokines in macrophages can cause a series of reactions, in turn causing continuous damage at the site of inflammation.[46,47] However, the MF extract inhibited the expression of these cytokines. It provides soothing relief for various problems from the skin inflammation.

During inflammation stages, macrophages perform several functions in wound healing: host defense, activating and repairing responses, and eating apoptotic cells.[48] Koh and DiPietro suggested that macrophage dysfunction drives the pathogenesis of non-healing or poorly healing wounds.[7] Thus, the macrophage is an attractive therapeutic target to reduce fibrosis and scarring and to improve chronic wounds. These might explain why anti-inflammatory property-possessing MF can also support the healing process in fibroblasts [Figure 5]. Among the five parts, MF has the most effective extract potentially in wound management and tissue remodeling.

CONCLUSION

In summary, the data demonstrated that MF most effectively protected skin cells against inflammatory damage, followed by MC, among the extracts of the five parts of *H. syriacus*. Although MF had low DPPH and ABTS scavenging activities, MF at 10 and 50 μg/mL increased hyaluronan secretion and decreased ROS production in HaCaTs and it decreased NO production as well as the expression of IL-6/IL-1 β and iNOS/COX-2. The better anti-inflammatory effects of MF can be partly explained by the higher contents of two active ingredients, palmitic acid and linoleic acid. It is necessary to assess the effect of MF and the underlying mechanisms *in vivo* in further studies.

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Conflicts of interest

There are no conflicts of interest.

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